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Mikuláš Klenor

Racionální návrh inhibitorů cyklin-dependentních kinas
Rational Design of Cyclin-Dependent Kinase Inhibitors

Bachelor's thesis

Advisor: prof. Ing. Pavel Hobza, DrSc., FRSC

Consultant: RNDr. Martin Lepšík, Ph.D.

Praha, 2021

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Statement of authorship

I declare that I am the sole author of this bachelor's thesis and I have stated all the used sources in the bibliography. Neither this work nor a substantial part of it has been submitted to gain a different or the same degree.

In Prague on May 6, 2021

Prohlášení

Prohlašuji, že jsem závěrečnou práci zpracoval samostatně a že jsem uvedl všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

V Praze, 6. května 2021

Mikuláš Klenor

Abstract

Cyclin-dependent kinases (CDKs) are enzymes which control the progression of eukaryotic cells through their cycle via binding to cyclins and phosphorylation. Any dysfunction in this process can lead to uncontrolled growth and thus development of severe diseases such as cancer. Their discovery was awarded by Nobel Prize in Physiology or Medicine in 2001 to Hartwell, Hunt and Nurse. Targeting CDKs with small-molecule inhibitors has shown practical utility as cancer therapy. In this Bachelor's thesis, the structure of CDK2, a prominent member of the family, is described and strategies to design small-molecule inhibitors are presented.

Keywords: Cyclin-dependent kinases, regulation, cell cycle, structure, inhibitor

Abstrakt

Cyklin-dependentní kinasy (CDK) jsou enzymy, které prostřednictvím vazby cyklinu a fosforylace kontrolují buněčný cyklus eukaryotických buněk. Jakákoliv dysfunkce v těchto procesech může vést k nekontrolovanému růstu a tedy k vývoji závažných onemocnění, např. rakoviny. Za objevy spojené s cyklin-dependentními kinasami byla roku 2001 udělena Nobelova cena za fyziologii a medicínu Hartwellovi, Huntovi a Nursovi. Inhibice CDK pomocí nízkomolekulárních látek slouží jako jeden ze způsobů chemoterapie rakoviny. V této bakalářské práci bude popsána struktura CDK2, modelového proteinu této rodiny, a poté budou ukázány strategie návrhu nízkomolekulárních inhibitorů CDK.

Klíčová slova: Cyklin-dependentní kinasy, regulace, buněčný cyklus, struktura, inhibitor

Content

1 Introduction

Regulation of all cellular functions to work as one well-tuned machine is a state which needs to be achieved by all multicellular living organisms. Protein kinases are group of proteins which play irreplaceable roles in signalization pathways which make this possible. This controlling network of a cell is encoded by more than 500 genes in the human genome – the so called kinome (Manning et al., 2002). Disorder in regulation of these enzymes can lead to severe health problems, for example cancer.

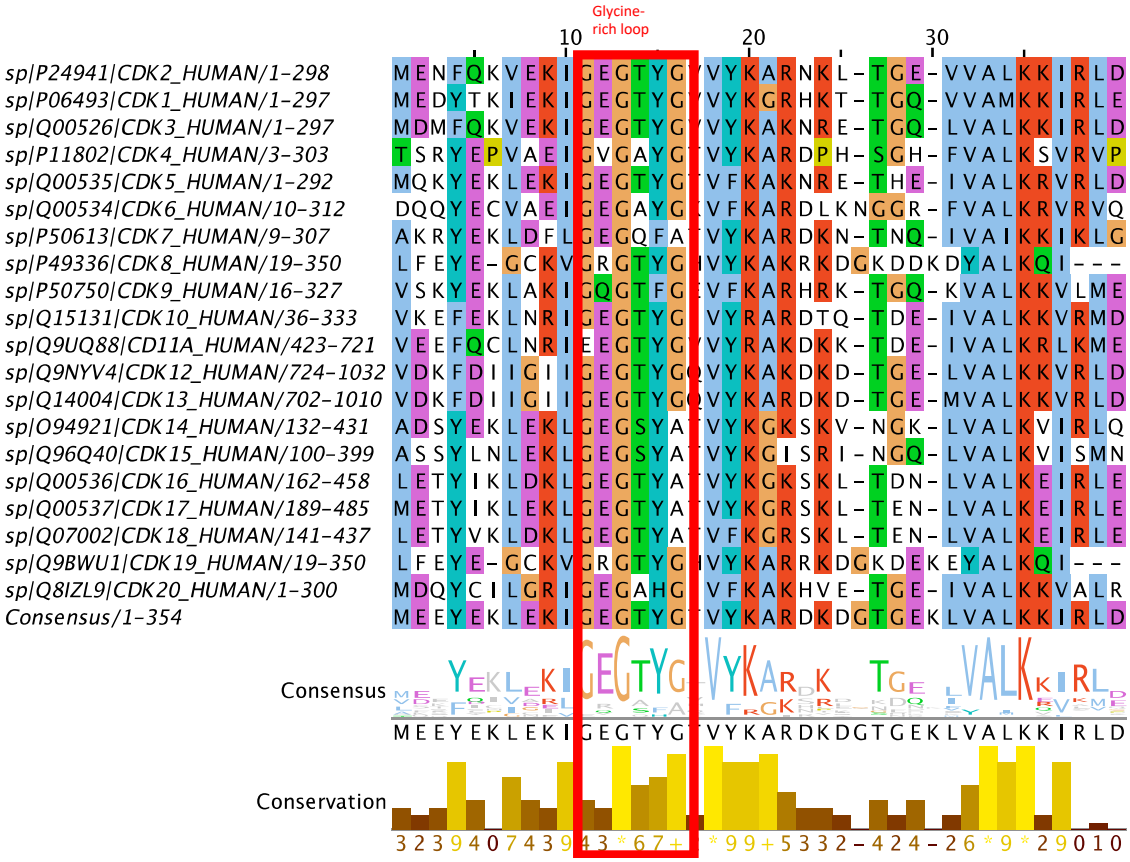
One subgroup of protein kinases is group of Cyclin-Dependent Kinases (CDKs). These enzymes, with their binding partner proteins called cyclins, are mainly responsible for the cell-cycle promotion but the spread of functions of these enzymes is much wider (Table 1). As CDKs evolved to be involved in many cellular functions, these enzymes are promising targets for different therapeutics, e.g. against cancer.

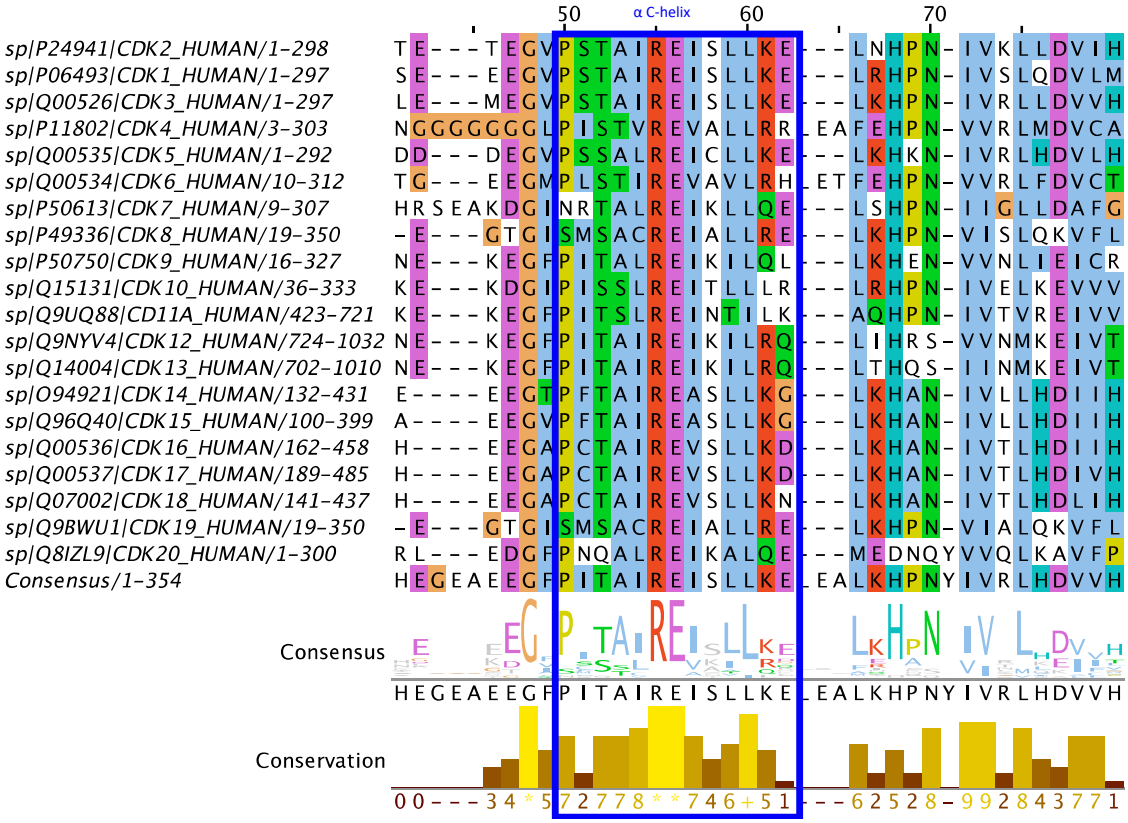
Table 1. Examples of functions of CDKs in humans

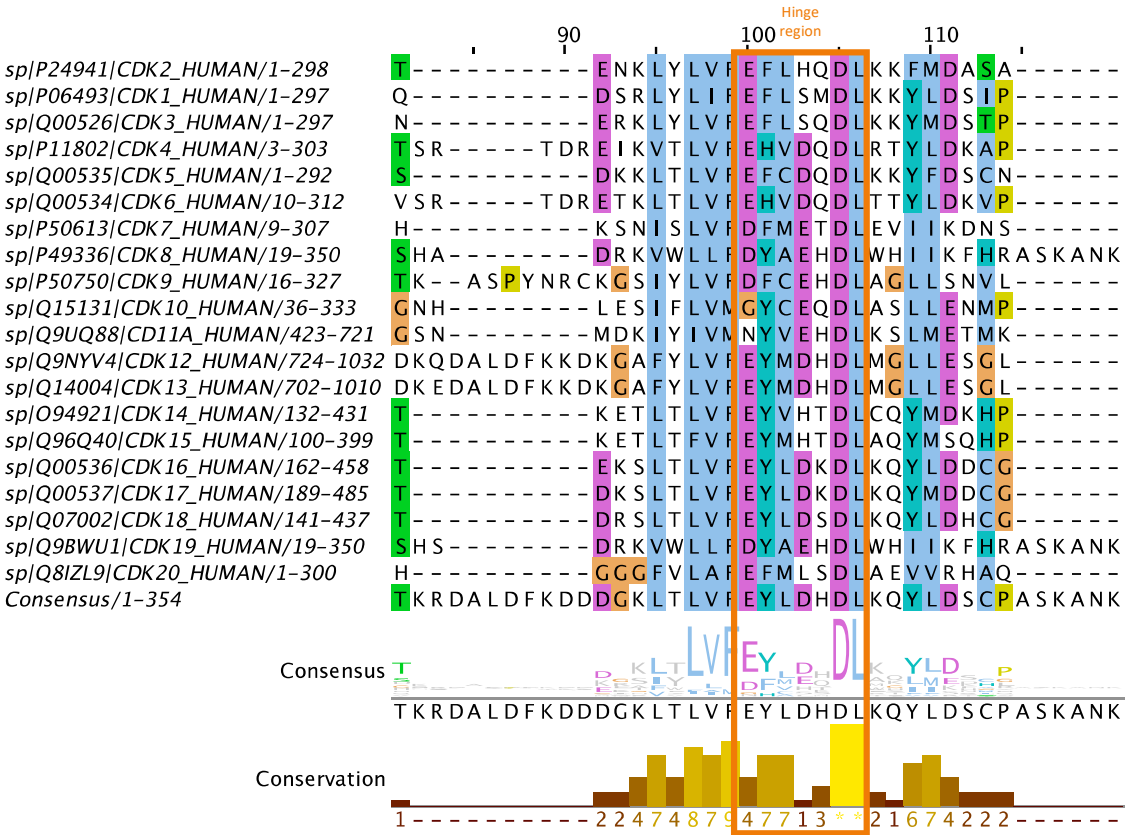
Protein	Function
CDK1	DNA replication regulation (S-phase) (Katsuno et al., 2009), mitochondrial activity regulation (G2 - M) (Z. Wang et al., 2014)
CDK2	G1 - S transition (Koff et al., 1992), DNA damage sensing (Huang et al., 2006), centrosome duplication (Meraldi et al., 1999), G2 - M check-point (Chung & Bunz, 2010)
CDK3	G0 exit (Ren & Rollins, 2004), G1 – S transition (Braun et al., 1998)
CDK4	G1 – S transition (Kato et al., 1993), embryonic development (Malumbres et al., 2004), development of pancreas (Rane et al., 1999)
CDK5	Brain development (Magen et al., 2015; Shinmyo et al., 2017), vesicle trafficking in synapsis (Ou et al., 2010)
CDK6	G1 - S transition (Meyerson & Harlow, 1994), embryonic development (Malumbres et al., 2004)
CDK7	Regulation of transcription (Glover-Cutter et al., 2009; Nilson et al., 2015)
CDK8	Regulation of transcription (Akoulitchev et al., 2000), embryonic development (Westerling et al., 2007)
CDK9	Regulation of transcription (Zhu et al., 1997), DNA damage repair (Yu et al., 2010)
CDK10	Actin fibers regulation (Guen et al., 2016), developmental regulation (Windpassinger et al., 2017)
CDK11	Autophagy regulation (Wilkinson et al., 2011), regulation of histone transcription during S-phase (Gajdušková et al., 2020)
CDK12	Regulation of transcription (S.-W. G. Cheng et al., 2012), DNA damage response (Blazek et al., 2011)

CDK13	Regulation of transcription (Fan et al., 2020), regulation of development (Sifrim et al., 2016)
CDK14	Noncanonical Wnt signalization (Sun et al., 2014), cellular mobility (Du et al., 2017)
CDK15	Apoptosis suppression (Park et al., 2014)
CDK16	Spermatogenesis (Mikolcevic et al., 2012), vesicle trafficking in synapsis (Ou et al., 2010)
CDK17	Brain development (Hirose et al., 1997)
CDK18	Development of Alzheimer's disease (Herskovits & Davies, 2006), DNA damage repair (Barone et al., 2016)
CDK19	Regulation of transcription (Sato et al., 2004), cellular stress response (Audetat et al., 2017)
CDK20	Hepato-cell carcinoma development (Feng et al., 2011)

Human CDKs have 292 (CDK5) - 1512 (CDK13) amino acids in length and share 37.8-76.4 % sequence similarity, taking as a reference the sequence of CDK2. They harbor common functionally important motifs (Fig. 1.A).




50
α C-helix
60
70
sp|P24941|CDK2_HUMAN/1-298
sp|P06493|CDK1_HUMAN/1-297
sp|Q00526|CDK3_HUMAN/1-297
sp|P11802|CDK4_HUMAN/3-303
sp|Q00535|CDK5_HUMAN/1-292
sp|Q00534|CDK6_HUMAN/10-312
sp|P50613|CDK7_HUMAN/9-307
sp|P49336|CDK8_HUMAN/19-350
sp|P50750|CDK9_HUMAN/16-327
sp|Q15131|CDK10_HUMAN/36-333
sp|Q9UQ88|CD11A_HUMAN/423-721
sp|Q9NYV4|CDK12_HUMAN/724-1032
sp|Q14004|CDK13_HUMAN/702-1010
sp|O94921|CDK14_HUMAN/132-431
sp|Q96Q40|CDK15_HUMAN/100-399
sp|Q00536|CDK16_HUMAN/162-458
sp|Q00537|CDK17_HUMAN/189-485
sp|Q07002|CDK18_HUMAN/141-437
sp|Q9BWU1|CDK19_HUMAN/19-350
sp|Q8IZL9|CDK20_HUMAN/1-300
Consensus/1-354
Consensus
HEGEAEEGFPITAIREISLLKELEALKHPNYIVRLHDVVH
Conservation


90
100
Hinge region
110
sp|P24941|CDK2_HUMAN/1-298
sp|P06493|CDK1_HUMAN/1-297
sp|Q00526|CDK3_HUMAN/1-297
sp|P11802|CDK4_HUMAN/3-303
sp|Q00535|CDK5_HUMAN/1-292
sp|Q00534|CDK6_HUMAN/10-312
sp|P50613|CDK7_HUMAN/9-307
sp|P49336|CDK8_HUMAN/19-350
sp|P50750|CDK9_HUMAN/16-327
sp|Q15131|CDK10_HUMAN/36-333
sp|Q9UQ88|CD11A_HUMAN/423-721
sp|Q9NYV4|CDK12_HUMAN/724-1032
sp|Q14004|CDK13_HUMAN/702-1010
sp|O94921|CDK14_HUMAN/132-431
sp|Q96Q40|CDK15_HUMAN/100-399
sp|Q00536|CDK16_HUMAN/162-458
sp|Q00537|CDK17_HUMAN/189-485
sp|Q07002|CDK18_HUMAN/141-437
sp|Q9BWU1|CDK19_HUMAN/19-350
sp|Q8IZL9|CDK20_HUMAN/1-300
Consensus/1-354
Consensus
TKRDALDFKDDDGKLTLVFEYLDHDLKQYLDSCPASKANK
Conservation

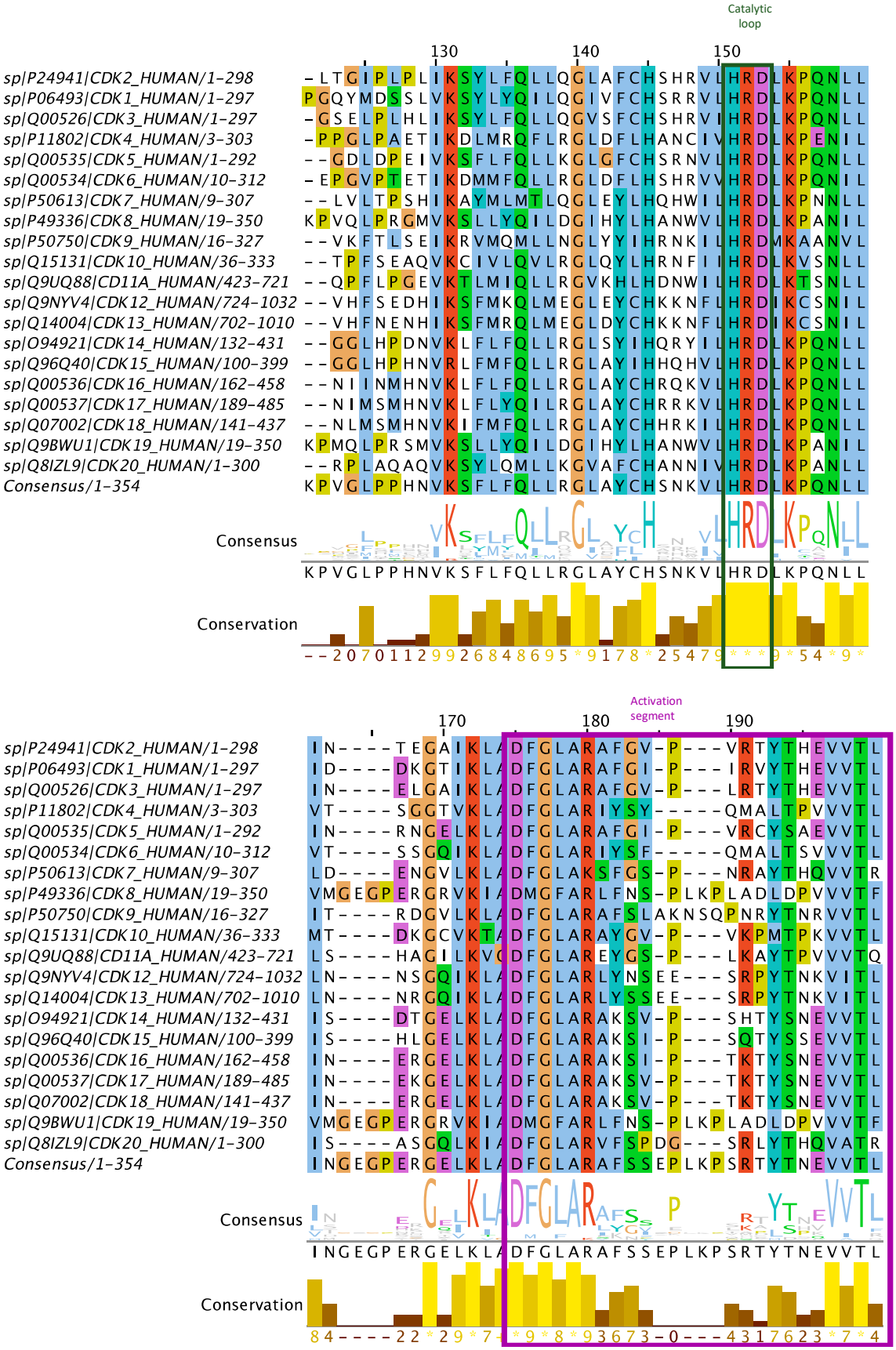


Fig. 1.A: Functionally important parts of 20 human CDKs shown in multiple sequence alignment. Prepared in Clustal Omega and Jalview (Sievers et al., 2011; Waterhouse et al., 2009); Red – Glycine-rich loop; Blue – αC-helix; Orange – Hinge region; Green – Catalytic loop; Magenta – Activation segment

2 CDK2

Out of the 20 human CDKs, CDK2 is by far the most thoroughly studied. We will thus use it for an exhaustive description of the structure. Other CDKs have similar structural features.

CDK2 plays pivotal role in the cell cycle regulation, mainly in G/S phase promotion (binding partner cyclin E) and during the S-phase (binding partner cyclin A) (Koff et al., 1992; Meraldi et al., 1999). Its full enzymatic capacity is only accessible when the respective cyclin binds and a specific phosphorylation site of the protein is phosphorylated (Stevenson et al., 2002).

Due to its crucial function in the cell division, CDK2 has been in the center of interest of designing small-molecule inhibitors, promising for their potential to become cancer therapeutics (Huwe et al., 2003). As an example, this effort can be expressed by the number of crystal structures recorded in the PDB database (www.rcsb.org) which is for CDK2 553 and the second most abundant is CDK1 with 157 entries as of April 2021.

2.1 Structure

In CDK2 structure, two main structural elements are distinguishable, a smaller N-terminal lobe (residues 1-83) and a larger C-terminal lobe (residues 84-298) (De Bondt et al., 1993). The lobes are linked by a flexible hinge region (residues 81-86). The N-lobe contains 5 antiparallel ß-strands and a larger α-helix (αC-helix), and the C-lobe dominantly consists of α-helices. The catalytic cleft is spatially oriented in between the two lobes and the hinge region and is covered by the glycine-rich loop (residues 10-16) and the activation segment (residues 145 – 170), an important regulatory site, which additionally accommodates residues of the Mg^{2+} binding site, the conserved DFG motif (residues 145 – 147) (Fig. 2.A) (De Bondt et al., 1993).

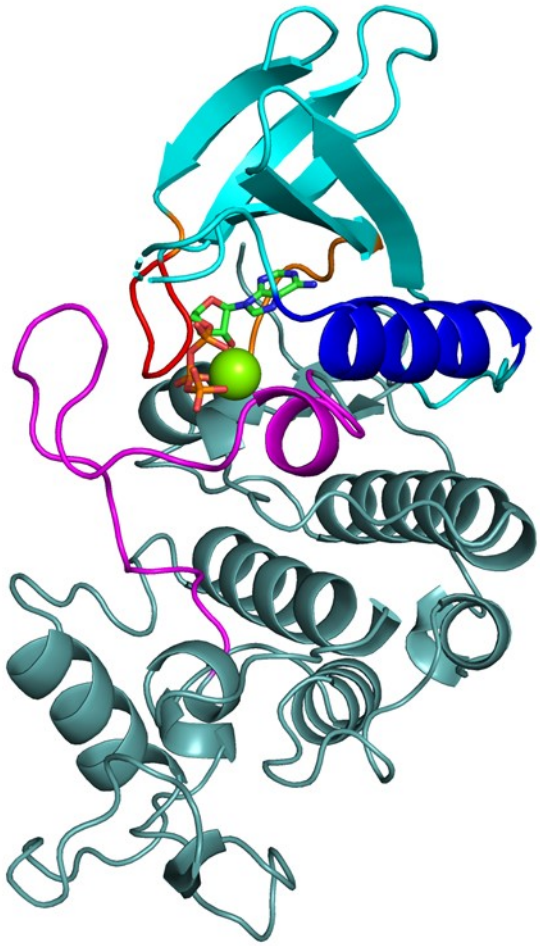


Fig. 2.A: Structure of CDK2; Light cyan - N-lobe; Dark cyan - C-lobe; Red - Glycine-rich loop; Blue - αC-helix; Orange - Hinge region; Magenta - Activation segment; coloured sticks – ATP (green – carbon, blue – nitrogen, red – oxygen), sphere Mg^{2+}; Colour scheme preserved in the following figures (PDB: 1HCK); For visualization used The PyMOL Molecular Graphics System, Version 2.1.1, Schrödinger, LLC.

Upon ATP binding, CDK2 forms 13 hydrogen bonds with the ATP molecule (Schulze-Gahmen et al., 1996). In addition, three water molecules bridge CDK2 with ATP. The Mg^{2+} ion is coordinated to one oxygen of each phosphate group of the ATP, to side-chain oxygens of Asn 132 and Asp 145 and, furthermore, to a water molecule inside the cavity. The adenine base interacts with the protein mainly by hydrophobic interactions. In addition, the backbone nitrogen of Leu 83 and backbone oxygen of Asp 81 in the hinge region make hydrogen bonds with nitrogen atoms of the adenine ring (Fig. 2.B). Due to hydrophobic environment, the interaction energy of these hydrogen bonds is higher compared to hydrogen bonds in a water solution, as they are not screened. The interacting nitrogen atoms of the adenine base take place of structural water molecules in the free enzyme(Schulze-Gahmen et al., 1996)

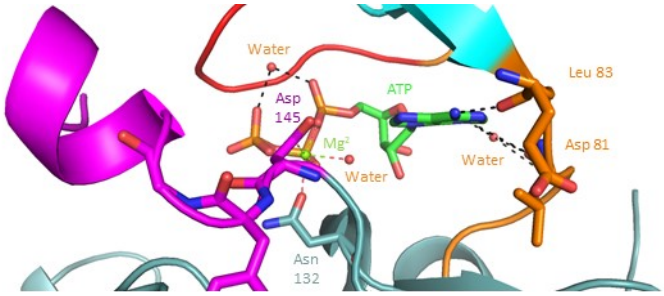


Fig. 2.B: Detail of the ATP-binding site of CDK2. Hydrogen bond network between ATP, Mg^{2+}, three water molecules, Asp 145, Asn 131, Asp 81 and Leu 83 (PDB: 1HCK)

The binding of ATP is not accompanied by significant changes in CDK2 conformation. (RMSD between the Cα atoms of the apoenzyme and the complex is small, 0.39 Å) (Schulze-Gahmen et al., 1996). Similar observations hold for conformations of side chains in the ATP-binding site. The only residues undergoing notable conformational changes are Lys 33, Lys 129 and Asn 132. In the case of Lys 33, the side chain NH_3^+ group loses a hydrogen bond with Asp 145 and with a water molecule in response to a shift towards the catalytic site (α-phosphate of the ATP). Lys 129 points towards Asp 127 in the apo-enzyme and, via its side-chain nitrogen, makes a hydrogen bond with the carboxyl oxygen of Asp 127. When ATP binds, this hydrogen bond is disrupted and the movement of Lys 129 results in a close contact of the residue with γ-phosphate of ATP (Fig 2.C and 2.D).

Furthermore, the binding of ATP introduces a conformational change of Asn 132 side chain which results in coordination of the Mg^{2+} ion. In the empty CDK2 structure, this hydrogen bond is found between Asn 132 and the backbone nitrogen of Lys 129 (Fig 2.C and 2.D) (Schulze-Gahmen et al., 1996).

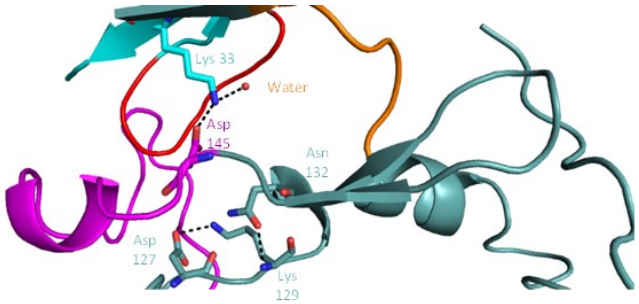


Fig. 2.C: Active cavity without ATP: Arrangement of interactions of Lys 33, Lys 129 and Asn 132 with their respective interaction partners: water molecule, Asp 145 and Asp 127 (PDB: 1HCL)

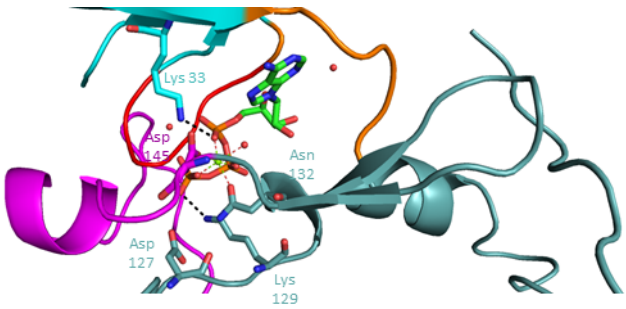


Fig. 2.D: Changes induced by ATP binding; Conformation changes of Lys 33, Lys 129 and Asn 132 in complex with ATP (PDB: 1HCK)

According to Liao et al. 2007, the catalytic cleft is divided into two areas – front pocket and back pocket (Liao, 2007). Front pocket comprises the ATP-binding site, the back pocket is separated by β8-strand, N-terminal part of the activation segment and by β3-strand (Liao 2007). Phe 80 (preceding the hinge region) plays a role of a gatekeeper (Liu et al., 1998), which controls the entrance to the back cavity (Liao, 2007).

2.1.1 Cyclin binding

The cyclin A/E subunits interact with both lobes of CDK2. On the side of the N-lobe, the main interacting site is the αC-helix. This interface is a rigid core of the CDK2/cyclin A interactions (Barrett & Noble, 2005). While cyclin A binds, αC-helix moves towards the catalytic site of CDK2 causing changes in conformation of the ß-strands of the N-lobe (Jeffrey et al., 1995). This interaction is a starting point leading to opening of the catalytic site. C-lobe/cyclin A interactions orchestrate movements of the activation segment by loosening the space in the vicinity of the segment. The induced changes in conformation of the activation segment are as large as 20 Å (Jeffrey et al., 1995; Morris et al., 2002). Conformational changes of the C-lobe and the activation segment are rate-limiting steps and are proposed to be involved in the selectivity for the respective cyclin (Morris et al., 2002).

CDK2/cyclin A dimerization partially activates CDK2 through induction of several interactions. It was shown that Arg 50 (part of the αC-helix) contributes to the activation by formation of hydrogen bond to the region of the Mg^{2+} stabilizing DFG motif (residue DFG + 2, Ala 149) (Kornev et al., 2006). Arg 150 interacts with the cyclin A subunit and, additionally, with Glu 162, part of the activation segment. These interactions mimic interactions of Arg 150 with phosphorylated Thr 160 and thus partially activate the CDK2 (Fig 2.E) (Kornev et al., 2006). Cyclin A binding increases the kinase activity to 0.4% of the fully activated form (Stevenson et al., 2002).

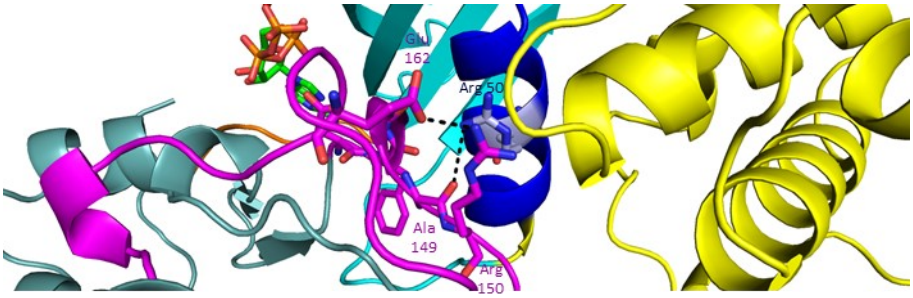


Fig. 2.E: Interactions induced by cyclin A; Coordination change of the DFG-motif and activation segment caused by interactions of Arg 50/Ala 149 and Arg 150/Glu 152; Colour scheme as previously, cyclin subunit in yellow (PDB: 1FIN)

Along with cyclin A, CDK2 is capable of binding cyclin E (Honda et al., 2005). Conformation of the complex and its interactions are similar to CDK2/cyclin A interface. The region where the CDK2/cyclin A and CDK2/cyclin E differ the most, is the C-terminal site of CDK2 (residues 289–297). The molecular surface area buried is increased by 14% in case of CDK2/cyclin E interface due to additional interactions of CDK2/cyclin E complex (Honda et al., 2005).

Even though the natural binding partner of Cyclin B is CDK1(Draetta et al., 1989; Z. Wang et al., 2014), cyclin B is capable to interact with CDK2 and activate the enzyme. CDK2/cyclin B interactions are weaker than those of CDK2/cyclinA/E (Nick R Brown et al., 2007).

2.1.2 Phosphorylation of CDK2

CDK2 is brought to the active conformation via phosphorylation by the CAK complex formed by CDK7, Cyclin H and MAT1 (Fisher & Morgan, 1994). The pivotal role in the phosphorylation is played by CDK7, which can additionally phosphorylate CDK1, CDK4, CDK6 (Larochelle et al., 1998; Schachter et al., 2013). CDK7 interacts with CDK2 in head-to-tail manner (Lolli & Johnson, 2007).

The activation segment of CDK2 (residues 145-170, magenta in Fig. 1.A and Fig. 2.A.) contains phosphorylation site conserved in most of the protein kinases. In case of CDK2, the phosphorylation takes place on Thr 160. Mutation of this residue substantially reduces the reaction rate of substrate phosphorylation (Adams et al., 1995). When Thr 160 is dephosphorylated, the activation segment occupies the entrance to the catalytic cleft, thus the phosphorylation acts as a key which unlocks the door of the ATP binding site resulting in increase in the ATP affinity, decrease of affinity for ADP and increase in substrate binding (N R Brown, Noble, Lawrie, et al., 1999; Stevenson et al., 2002). As in case of the partial activation of CKD2 by cyclin A binding, phosphorylated CDK2 without cyclin bound has low detectable enzymatic activity (0.3% of fully activated CDK2).

Upon phosphorylation, Thr160 moves 6.1 Å from the solvent-exposed area (near the catalytic cleft) towards the CDK2/cyclin A interface and introduces several van der Waals interactions between hydrophobic residues of the activation segment and cyclin A (Russo et al., 1996). Residues of CDK2 providing these interactions are Val 154 and Pro 155. Phosphorylation thus does not negatively affect the affinity for CDK2/cyclin A binding (N R Brown, Noble, Lawrie, et al., 1999).

The negative charge of the phosphate group of pThr160 is neutralized by three Arg residues (Arg 50, Arg 126 and Arg 150) (Fig 2.F) (Barrett & Noble, 2005; N R Brown, Noble, Endicott, et al., 1999; Honda et al., 2005; Russo et al., 1996). The low dielectric constant of the environment surrounding pThr160 increases the interaction energy of the three Arg residues due to reduced screening (Russo et al., 1996). The interacting Arg residues are parts of conserved structural and sequence elements. Arg50 is part of the αC-helix PSTAIRE motif (residues 45-57 in CDK2 sequence), Arg 126 contributes to the HRD motif in the catalytic site (Bao et al., 2011) and Arg 150 is preceding the Thr 160 in the sequence of the activation segment (Russo et al., 1996). In addition to the coordination of pThr160, Arg 50 and Arg 150 make hydrogen bonds to cyclin A, the HRD motif and orient the Mg2+ binding DFG motif (Russo et al., 1996).

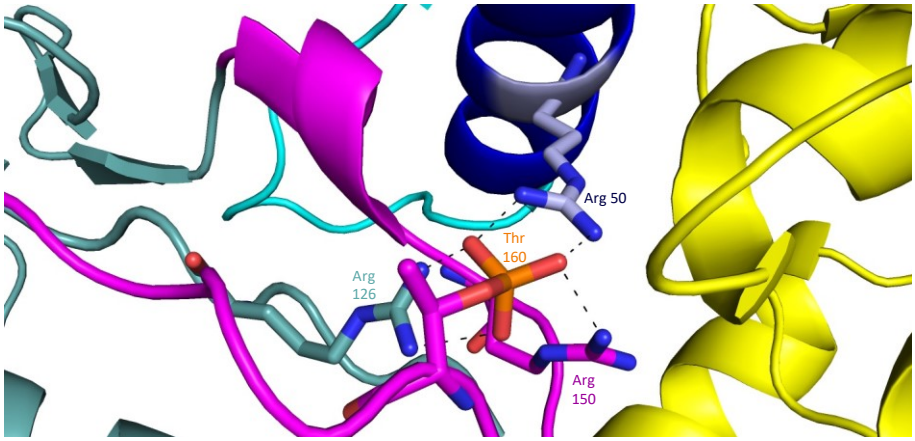


Fig. 2.F: Neutralisation of negative charge on pThr 160; Arg 50, Arg 126 and Arg 150 interact with pThr 160 (PDB: 1WNZ)

As shown in a molecular dynamics study, the main driving force for the movement of pThr 160 towards the arginine triad is the charge-charge interaction, but the contribution of each Arg of the triad is not equal (Barrett & Noble, 2005). Authors of the study performed molecular dynamics simulations of mutated CDK2 structures (R50M, R126M and R150M). The most significant disruption of the triad/pThr160 interactions was in the case of R126M, showing the inequality in the stabilization.

The phosphorylation causes structural changes in which the peptide bond between residues in the vicinity of the DFG motif (peptide bond between residues DFG + 1 and DFG + 2, Leu 148 and Ala 149, respectively) turns toward Arg 126 (Kornev et al., 2006).

Apart from the activation segment, a second regulatory site occurs in CDK2. The glycine-rich loop, located in the N-lobe, contributes to the right orientation of ATP in the binding site and by phosphorylation, this segment is involved in inhibition of CDK2 function (Bártová et al., 2004).

2.1.3 Conserved structural elements

Structural conservation of the ATP-binding site across the kinome allowed a classification of protein kinase conformations based on the conformation of αC-helix and the DFG-motif. There are five

groups of combinations of the αC-helix and the DFG-motif (Fig 2.G): CIDI (active conformation) – αC-helix in, DFG-motif in; CODI - αC -helix out, DFG-motif in; CIDO - αC-helix in, DFG-motif out; CODO - αC-helix out, DFG-motif out; ωCD – other conformations of αC-helix and DFG-motif (Ung et al., 2018).

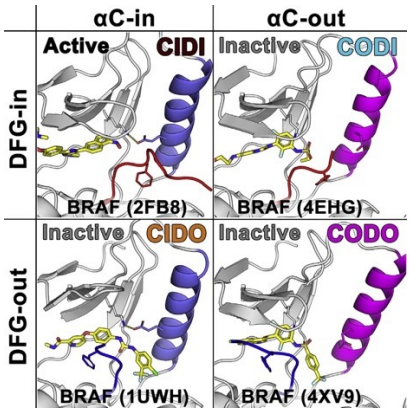


Fig. 2.G: Different conformations of αC-helix and the DFG-motif which define conformations of protein kinases (Figure adopted from Ung et al., 2018)

2.2 Substrate phosphorylation

One of the natural substrates of CDK2 is retinoblastoma protein (Rb) which is involved in the pRb/E2F cell-cycle progression (Akiyama et al., 1992)

The optimal substrate sequence for CDK2/cyclin A phosphorylation is Ser/Thr-Pro-X-Lys, where the Ser/Thr residue is the site of phosphorylation (Songyang et al., 1994). CDK2 preferentially phosphorylates substrates with Pro residue at the P + 1 position (one position after the phosphorylated Ser in the sequence) and with a basic amino acid at P + 3 (Songyang et al., 1994; Stevenson-Lindert et al., 2003). Basic amino acids tend to precede the phosphorylation site.

The basis for the preference for Pro in P + 1 position arises from the favorable orientation of the activation segment. The activation segment forms a pocket which accommodates the P + 1 Pro residue (N R Brown, Noble, Endicott, et al., 1999). In the dephosphorylated form of CDK2/cyclin A complex, this pocket is occupied by main chain oxygen of Val 163 of CDK2. Motions coupled with the phosphorylation of Thr160 of CDK2 influence the orientation of Val 163, letting the Pro P + 1 enter the catalytic space (N R Brown, Noble, Endicott, et al., 1999).

The correct adjustment of the activation segment and the catalytic loop is possible due to establishment of hydrogen bond network which spreads from Val 164 (activation segment), Arg 169 (activation segment) to Arg 126 (catalytic loop). By these interactions, Val164 obtains naturally less stable conformation ($\varphi = 72.5°$, $\psi = 130.8°$). Furthermore, Pro, as an imino acid, does not need additional hydrogen bond to compensate the polar hydrogen of nitrogen in the main chain of regular amino acids. This effect determines the exclusivity for Pro on P + 1 position (Fig 2.H) (N R Brown, Noble, Endicott, et al., 1999).

Likewise, Pro in position P + 1, preferentially basic residues occur on the position P + 3 positively contributing to the formation of the CDK2/cyclin A/substrate complex. The main interactions, which provide this relationship, stem from interactions of phosphate of pThr 160 bridging the P + 3 basic residue and Ile 270 of cyclin A.

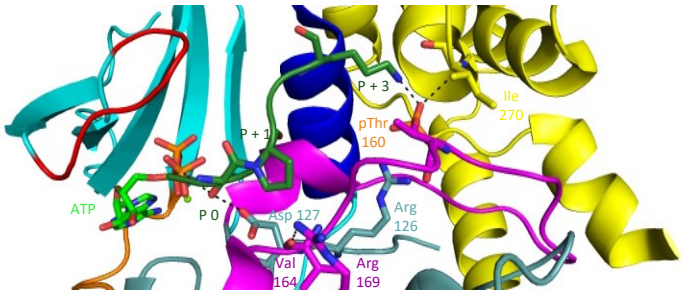


Fig. 2.H: Active cavity with bound substrate; Interaction network of Val 164, Arg 169 and Arg 126 which orients the catalytic residue Asp 127 with the reacting, dephosphorylated P 0 Ser and ATP molecule; Interactions pThr 160 phosphate bridging P + 3 basic residue of substrate and Ile 270 of cyclin A (PDB: 1QNZ)

In the list of substrates phosphorylated by CDK2 occur substrates which do not contain the ideal sequence mentioned previously. For these cases, the way for the reaction is cleared by interaction of conserved RXL motif of the substrate with the cyclin subunit (K.-Y. Cheng et al., 2006; Stevenson-Lindert et al., 2003; Takeda et al., 2001). The RXL motif is not only responsible for binding of substrates, some peptide inhibitors are capable of binding the CDK2 through this motif (J. Chen et al., 1996). The success of phosphoryl-transfer depends on the distance of RXL motif from the phosphorylation site (K.-Y. Cheng et al., 2006). The minimal distance for the reaction to be processed is 17 residues from the P 0 Ser residue.

On the side of the CDK2/cyclin A complex, the RXL motif interacts mainly with the cyclin subunit (N R Brown, Noble, Endicott, et al., 1999). These interface increases local concentration of the substrate and therefore lower the barrier for the high reaction rate(K.-Y. Cheng et al., 2006).

CDK2/cyclin B/substrate interface does not depend on the recognition of RXL motif which results into a potential of CDK2/cyclin B complex to regulate proteins that do not comprise the RXL motif and are not recognized by CDK2/cyclin A complex (Nick R Brown et al., 2007).

2.2.1 Reaction mechanism

Two models for CDK2 phosphoryl-transfer reaction mechanism were proposed: i) the hydrogen of the phosphorylated Ser of the substrate is transferred directly to the γ-phosphate of the ATP (Cavalli et al., 2003), and ii) the hydrogen transfer occurs via Asp 127 residue (Fig 2.I)(N R Brown, Noble, Endicott, et al., 1999). As indicated by recent studies (Bao et al., 2011; Recabarren et al., 2019; Smith et al., 2011) the model of Asp127 acting as a general base in the catalysis is more probable and thus will be further discussed.

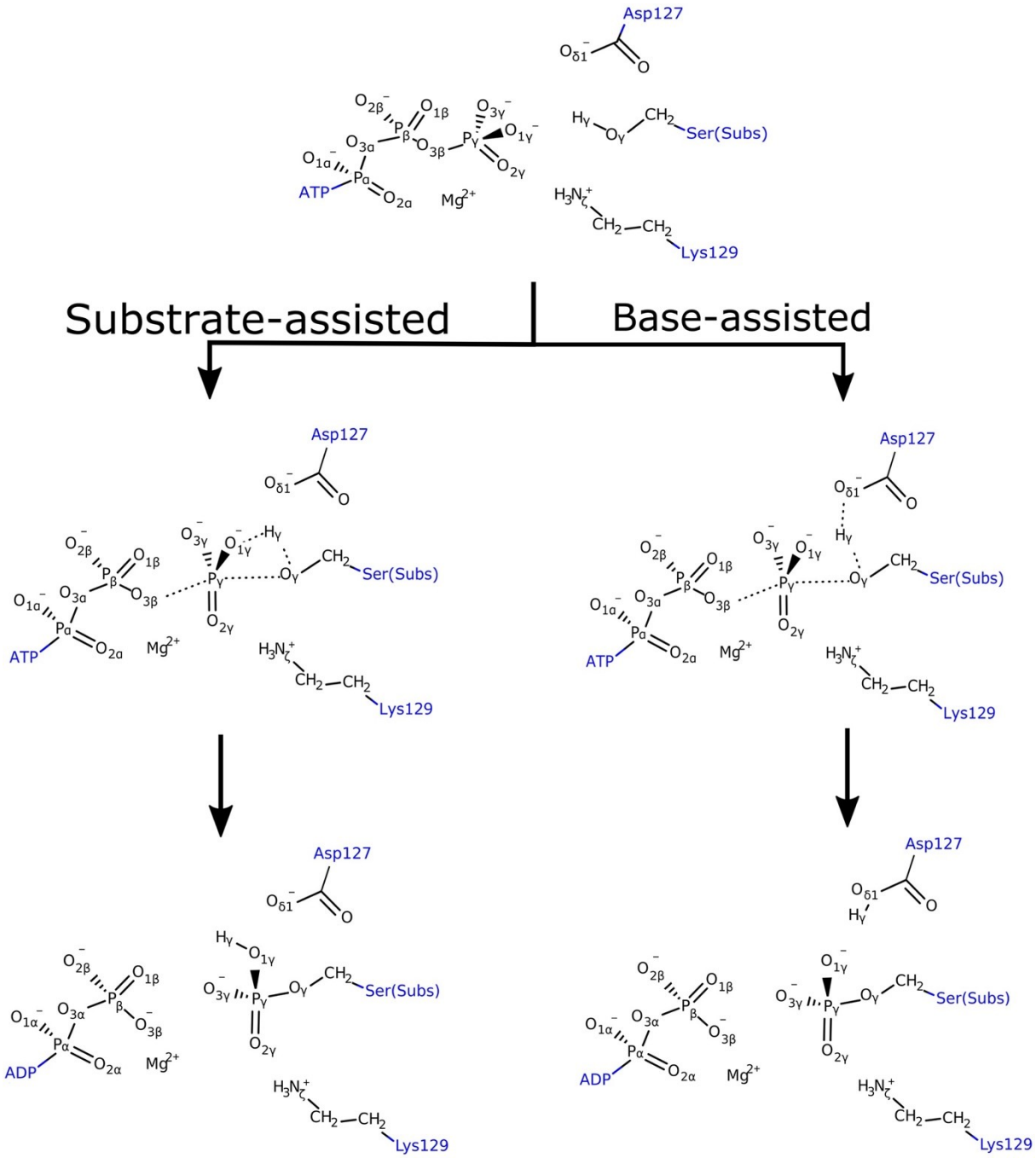


Fig. 2.I: Scheme of reaction mechanism of CDK2 phosphorylation; On the left side, γ-phosphate acts as a base which interacts with hydrogen atom of P 0 Ser residue. As a response P 0 Ser attacts the γ-phosphate with its oxygen atom resulting in phosphorylation of P 0 Ser; On the right side, Asp 145 interacts with hydrogen atom of P 0 Ser. This contact results in attack of the γ-phosphate of ATP by P 0 Ser; In both cases, Lys 129 stabilizes the negative charge of the γ-phosphate; (Recabarren et al., 2019)

In the catalytic complex, the P 0 Ser is in contact with the γ-phosphate of ATP. The lone pair of electrons of the oxygen atom of Ser P 0 is positioned in-line with the β-/γ- phosphate bond (N R Brown, Noble, Endicott, et al., 1999). In this arrangement, Asp 127 takes the proton from Ser P 0 (N R Brown,

Noble, Endicott, et al., 1999; Recabarren et al., 2019; Smith et al., 2011). The transition state is stabilized by Lys 129 which interacts with the negatively charged γ- phosphate. Additionally, Lys129 exchanges a proton with the transferred phosphate during the transition state when Asp127 receives a proton from the substrate Ser (Fig. 2.I) (Recabarren et al., 2019). In this model, one Mg^{2+} ion is present, structurally arranged as described previously.

A model of two Mg^{2+} ions placed in the catalytic cavity during the phosphoryl-transfer was recently proposed (Bao et al., 2011; Jacobsen et al., 2012). During the transition state the glycine-rich loop collapses into a closed conformation which sandwiches the phosphate groups of the ATP and provides main chain amides of the glycine-rich loop o interact with the oxygen atoms of the ATP phosphate. This formation makes the complex more rigid compared to the scheme of one Mg^{2+} ion bound (Bao et al., 2011).

The stiffness of the complex results in the stabilization of the transition state and leads to the progress of the reaction. In addition, the second Mg^{2+} does not occur in the thermodynamically ideal conformation/coordination. Interaction scheme of the residues in the active site is similar to that described in the model of one Mg^{2+} ion (Bao et al., 2011).

The question of which model is correct can be further studied by quantum mechanical computational methods which provide a great insight into the picosecond-scaled processes, e.g. chemical reactions.

3 Inhibition

Most of CDKs share a significant sequence similarity (Fig. 1.A) which is apparent also as a structural similarity especially in the active site. As a result, inhibitors interact with CDKs in a similar fashion and specificity is difficult to obtain.

Three generations of CDKs inhibitors have been already proposed. Inhibitors of first generation have multi-CDK specificity. Representative compound of this group is Flavopiridol (Sedlacek et al., 1996). Like many of other first-generation inhibitors, its development was stopped for failing to pass the licensing rules (Blum et al., 2011). Second generation of CDK inhibitors sought higher protein specificity, mainly towards targeting CDK1 and CDK2. The representative compound of this class is Dinaciclib (Parry et al., 2010). Dinaciclib did not achieve beneficial advantages for use and its development was stopped (Stephenson et al., 2014). None of second-generation inhibitors is approved for use in therapy but some of the compounds are still in development. The third generation of CDK inhibitors was focused on development of CDK4/6 selective inhibitors. This effort led to the design of the first FDA-approved (The United States Food And Drug Administration) small-molecule inhibitors of CDKs: Palbociclib, Abemaciclib and Ribociclib (Finn et al., 2016; Hortobagyi et al., 2016; Patnaik et al., 2016)

CDK inhibitors are divided into 7 groups based upon specific interaction sites of these inhibitors. Type I, type I½, type II and type III inhibitors bind to the ATP-binding site or in its vicinity. Type IV inhibitors bind to allosteric pockets far from the ATP-binding site, type V inhibitors bind to two binding sites or to two different proteins and type VI inhibitors are molecules which bind covalently to the protein. This differentiation was developed by authors of several reviews (Dar & Shokat, 2011; Gavrin & Saiah, 2013; Lamba & Ghosh, 2012; Roskoski, 2016; Zuccotto et al., 2010).

3.1 Type I and I½ (DFG-in inhibitors)

Type I inhibitors bind to the ATP-binding site in the active conformation of the protein kinases (CIDI). Phe residue of the DFG-in motif occupies the back cavity formed in the DFG-out conformation (CIDO/CODO) . As a result of the structural similarity to ATP-binding, type I inhibitors are competitive inhibitors (Ung et al., 2018).

CODI conformation is preferred by type I½ inhibitors (Ung et al., 2018). The partially disordered activation segment interacts with the αC-helix and, as a response of the contact, unlocks the entrance to the back pocket formed by the DFG-out conformation (Ung et al., 2018).

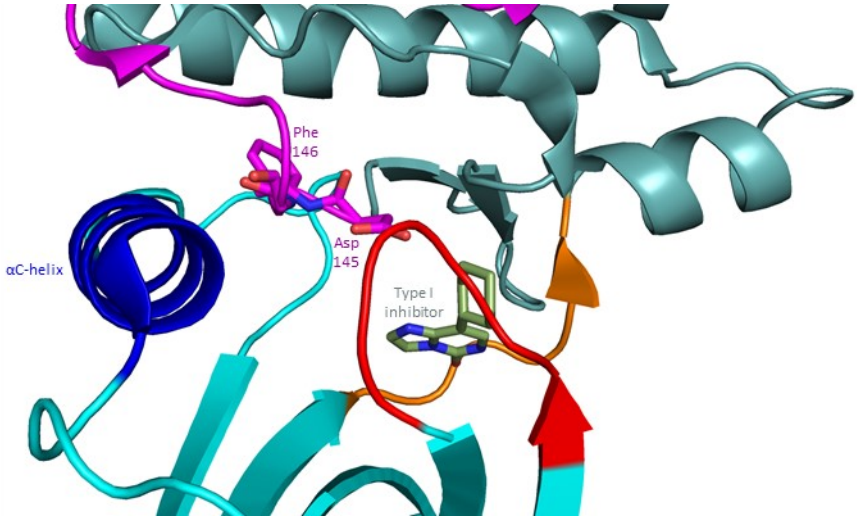


Fig. 3.A: DFG-in inhibitor in complex with CDK2; In blue shown conformation of αC-helix, in magenta the DFG-motif, green sticks – type I inhibitor (PDB: 7ACK)

Tutone et al. 2020 proposed a pharmacophore for ATP-competitive inhibitors as a compound containing H-bond acceptors and donors, hydrophobic moiety and an aromatic ring (Tutone et al., 2020). Specific attributes of volume, depth and hydrophobicity of the cavity are required from the protein to accommodate type I inhibitors (Volkamer et al., 2015).

As a result of high conservation of the protein kinase domain, frequent problem of type I inhibitors is low selectivity. To generate more selective protein kinase inhibitors, a promising approach is to target the αC-helix out conformation (Kwarcinski et al., 2016).

3.2 Type II

Protein kinase conformation for binding type II inhibitors is well conserved among the kinome (Vijayan et al., 2015) and can be characterized as CIDO conformation (Ung et al., 2018). As opposed to CIDI, in CIDO conformation, the DFG motif is reshaped into the DFG-out conformation, which leads to formation of a binding cavity for accommodation of type II inhibitors. The activation segment adopts an inactive conformation and can form secondary structure elements (Ung et al., 2018).

Type II inhibitors interact with the cavity by hydrogen bonding formed to the main chain amide of Asp residue of the DFG motif and carboxylate of Glu residue of αC-helix (Fig. 3.B) (Ung et al., 2018).

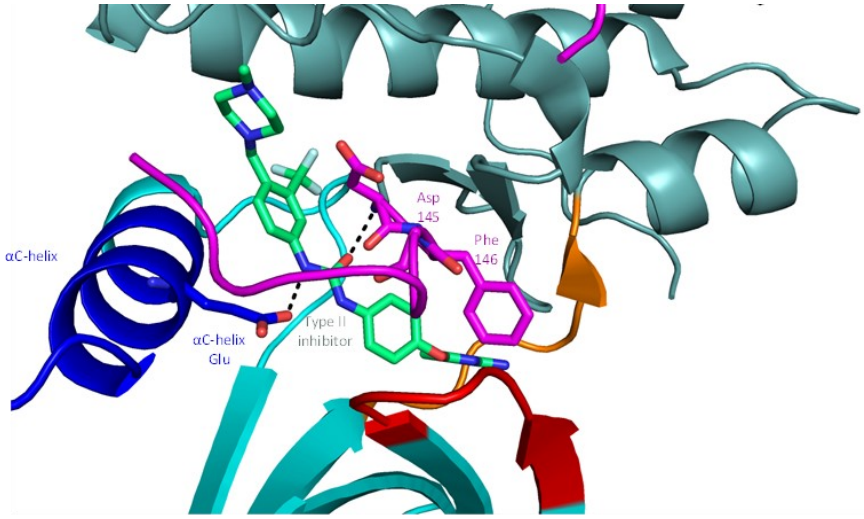


Fig. 3.B: Type II inhibitor in complex with CDK2; Inhibitor interacting with Asp 145 and Glu 51(PDB: 5A14)

The pharmacophore for type II inhibitors consists of an amide-type linker, which is capable of forming hydrogen bonds to Asp residue of the DFG motif and to Glu residue of the αC-helix. Secondly, type II inhibitors contain a substituted aryl moiety, which binds to the DFG pocket (Ung et al., 2018). Along with these observations, CDK can bind type II inhibitors in a similar arrangement. Compared to other protein kinases, the DFG-out conformation is disrupted by the binding of cyclin subunit (Alexander et al., 2015).

Partially as a result of success in designing selective first-generation type II inhibitors inhibiting, for example, Abl kinase (Cumming et al., 2004; Jung et al., 2006; Wilson et al., 1997), it was believed that type II inhibitors are more selective than type I inhibitors. To elucidate this, Vijayan et al. 2015 exhibited profiling assay of 9 type II inhibitors against a panel of 350 protein kinases (Vijayan et al., 2015). Results of this study, combined with previously published profiling assay (Anastassiadis et al., 2011), showed that type II inhibitors are more selective than type I. On the other hand, Kwarcinski et al. 2016 carried out experiments with type I/type II analogues. In conclusion, this study showed that type II inhibitors can be less selective than type I inhibitors (Kwarcinski et al., 2016).

3.3 Other types of inhibitors

In comparison to type I and type II inhibitors, allosteric and bisubstrate inhibitors have an advantage of higher specificity. In case of allosteric inhibition, targeted pockets are less conserved than the ATP-binding site throughout the kinome (H. Wang et al., 2017). This observation might originate from slightly or largely different regulatory mechanisms of each protein kinase.

Type III inhibitors bind to a cavity located in between the activation segment, which is in its inactive, disordered state, and the αC-helix (in out conformation) (Fig 3.C) (Betzi et al., 2011; Rastelli et al., 2014a). The allosteric cavity is found near the ATP-binding site. Interactions of type III inhibitors

induce structural changes which prevent binding of the cyclin subunit (Betzi et al., 2011; Christodoulou et al., 2017; Rastelli et al., 2014b).

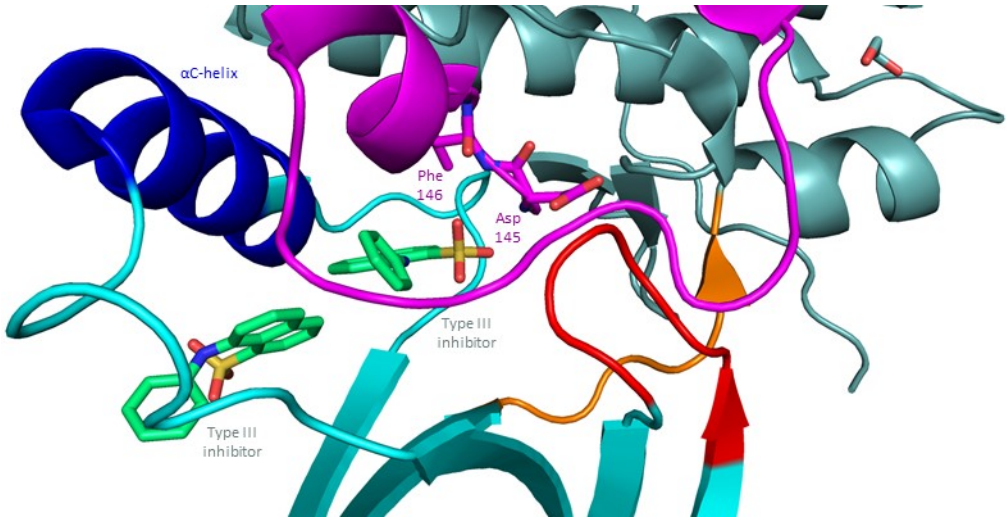


Fig. 3.C: Type III inhibitor in complex with CDK2; Two molecules of type III inhibitor (green sticks) interacting with the αC-helix and the DFG-motif (PDB: 3PXF)

Several attempts to inhibit CDK by interactions with this allosteric pocket have been tried. Disadvantage of all of the designed compounds is the lack of affinity (Betzi et al., 2011; Carlino et al., 2018; Rastelli et al., 2014b).

Type IV inhibitors bind to an allosteric cavity which is distant from the ATP-binding site. Promising site to be targeted by type IV inhibitors is the CDK/cyclin interface. As in case of type III inhibitors, type IV inhibitors lack higher affinity for the protein (Hu et al., 2015). Lamba et al. 2012 explains the low affinity as a result of shallowness of the protein/protein interface which causes incapability of tight interactions between the small molecule and the inhibited protein.

In recent years, a new approach of Proteolysis Targeting Chimeras (PROTACs) has emerged. This strategy is based on recruitment of E3 ligase to the target protein by a small, bivalent molecule leading to metabolization of the target in proteasome (Olson et al., 2018; Rana et al., 2019; Riching et al., 2020; Wei et al., 2021; Zhou et al., 2020)

Another potent approach to inhibit CDKs is to form a covalent bond to the enzyme. This enables targeting shallow protein cavities (Craven et al., 2018). Residues undergoing covalent bonding are mainly Cys residues (Gao et al., 2018; Kwiatkowski et al., 2014; Zhang et al., 2016) or Lys residues (Anscombe et al., 2015). Reversible variants of covalent inhibitors are frequently less potent than the irreversible covalent variants (Olson et al., 2019; Zhang et al., 2016).

Interesting history has evolved for covalent inhibitor THZ1 which inhibits selectively CDK7 (Kwiatkowski et al., 2014). THZ1 specifically covalently binds to non-conserved Cys residue in the vicinity of the ATP-binding site. Further development led to discovery of covalent inhibitor of CDK12

and CDK13 (Zhang et al., 2016). THZ1 was later considered as substrate of ABC1 or ABC2 proteins which induce resistance to this compound, therefore, authors of the study proposed a compound less sensitive to drug resistance than THZ1 (Gao et al., 2018).

Another cavity to be targeted in case of CDK2 spreads from residue 169 to residue 298 in the C-lobe. As a good starting point, a unique Cys 177 is located in this area (Craven et al., 2018).

3.4 FDA-approved inhibitors of CDKs

There are three FDA-approved CDK inhibitors, Palbociclib, Ribociclib and Abemaciclib (https://www.fda.gov). All three inhibitors can be used in treatment of HR+ (hormone receptor positive) breast cancer and are in clinical trials for treatment of other types of cancer disease. All the inhibitors inhibit CDK4 and CDK6 and correspond to inhibitors of type I½. Additionally, in complex of CDK6 and cyclin D, Palbociclib adopts conformation of type I inhibitor (Lu & Schulze-Gahmen, 2006).

All three inhibitors bind to the ATP-binding site and interact with the hinge region (P. Chen et al., 2016). A water molecule is visible in the structure of CDK6/Ribociclib complex buried inside the hinge region. Position of the molecule is adjacent to the Ribociclib and His 100 of the CDK6 (Phe 83 in CDK2) and is suitably arranged to mediate interactions of Ribociclib and the His residue. In structures of the other two inhibitors, this water molecule is not found. From orientations of the inhibitors and His 100, it is likely that the water molecule is present in a similar arrangement as in the CDK6/Palbociclib-Abemaciclib complexes. The binding of Ribociclib to CDK6 induces small distortions to the glycine-rich loop. Abemaciclib is bound more deeply in the cavity compared to Palbociclib/Ribociclib and, additionally, interacts with Lys 43 (analogous to CDK2's Lys 33) (P. Chen et al., 2016).

In complex of CDK6/Cyclin/Palbociclib, Palbociclib interacts through 73 contacts with the CDK6, including 4 hydrogen bonds (Lu & Schulze-Gahmen, 2006). Two hydrogen bonds are formed to Val 101 (CDK2 Leu 83) and additional hydrogen bonds are found between Palbociclib and Asp 102 (CDK2 His 84) and Asp 163 (CDK2 145) (Lu & Schulze-Gahmen, 2006).

Selectivity of Palbociclib, Ribocliclib and Abemaciclib may be governed by substitution of residues in the hinge region (His 100 CDK6 vs. Phe 82 CDK2 and Phe 39 CDK6 vs. Val 29 CDK2) and steric clashes in the hinge region caused by the gatekeeper residue (CDK2 Phe 80) (P. Chen et al., 2016; Lu & Schulze-Gahmen, 2006). Additional region, which ensures specificity of these inhibitors, is the mouth of the ATP-binding site (Lu 2006, Chen 2016). Residue Thr 107 is, in case of CDK 1/2/3/5, substituted for Lys (CDK2 Lys 89). This area is solvent exposed (P. Chen et al., 2016; Lu & Schulze-Gahmen, 2006).

4 Conclusion

As aging is one of the problems of today's world, cancer treatment is a field of interest which has gained much more importance than ever before. Cyclin-dependent kinases are promising targets due to their role in the cell cycle. Many attempts to target these enzymes with different approaches have been carried out over the last 20 years but this effort was successful only in few cases. Palbociclib, Abemaciclib and Ribociclib are the first FDA-approved CDK inhibitors, which can be used for the treatment of breast cancer. As these compounds are used in the clinics, new problem of resistance towards these compounds has emerged, revied in Pandey et al., 2019 (Pandey et al., 2019). This is an example of demand for continuous development of selective inhibitors of CDK and other protein kinases. Apart from cancer treatment the use of CDK inhibitors extends our knowledge of cellular mechanisms underlying basics of existence of life.

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